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Genetic Variation in the Apogamous Fern *Cyrtomium fortunei* (Dryopteridaceae)

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In apogamous ferns, all offspring from a parent are expected to be clonal. However, apogamous species frequently show a large amount of morphological and genetic variation. *Cyrtomium fortunei* and its relatives, which are distributed throughout Japan, are reported to be apogamous triploids, but show large and continuous morphological variation. Four varieties of *C. fortunei* have been recognized. We sought to determine whether an apogamous species has genetic variation, and if so, whether this variation relates to morphological variation within local populations. Among 224 individuals growing in four distantly located populations in Japan, where several varieties grow together, two *rbcL* types (α and β) and eight allozyme types (A–H) were identified. Several different genetic clones were detected in the local populations examined. Only individuals that could be morphologically identified as *C. fortunei* var. *intermedium*, based on bicolored indusia, had *rbcL* β and allozyme type H, and thus, were genetically differentiated by their nuclear and plastid genomes from the other three varieties of *C. fortunei*. The other three varieties shared the same *rbcL* (α), making correlation between allozyme types and varieties, especially genetic differences between var. *fortunei* and var. *clivicola*, unclear.

Key words: allozyme, apogamy, clonal diversity, *Cyrtomium fortunei*, genetic variation, population genetics, *rbcL*

Apogamy, in the broad sense, or agamospory in ferns, is a type of asexual reproduction in which unreduced spores are formed. The resultant gametophytes produce sporophytes of the next generation without fertilization (Manton 1950). Apogamous reproduction is common in ferns. Approximately 10% of the world's fern species (Lovis 1977) and 15% of Japanese fern taxa have apogamous reproduction (Takamiya 1996). In apogamous ferns, all offspring from a sporophyte are genetically the same unless mutations occur during reproduction. The amount of genetic variation within an apogamous species is therefore expected to be very low, especially in apogamous species not of recent and recurring origin.

Darnaedi *et al.* (1990) reported the absence of allozyme variation within *Dryopteris yakusilvicola* Sa. Kurata, a triploid apogamous species of recent hybrid origin endemic to Yaku Island. Absence of allozyme variation, however, is uncommon. In spite of the clonal nature of apogamous reproduction, many apogamous fern species show large morphological and some genetic variation (Watano & Iwatsuki 1988, Suzuki & Iwatsuki 1990, Lin *et al.* 1995, Takamiya *et al.* 2001). Previous studies analyzing genetic variation in apogamous ferns using enzyme electrophoresis reported 45, 4, and 14 different clones from *D. nipponensis* (Ishikawa *et al.* 2003a), *Asplenium unilaterale* (Watano & Iwatsuki 1988), and *D. bissetiana* (Lin *et al.* 1995), respectively. Further-

more, genetic variation in combination with cytological variation has been reported in apogamous ferns. Six different triploid and five diploid clones have been reported in *Pteris cretica* (Suzuki & Iwatsuki 1990) and one tetraploid and four triploid clones have been reported in *Diplazium doederleinii* (Takamiya *et al.* 2001).

Cyrtomium C. Presl (Dryopteridaceae), characterized by peltate indusia and anastomosing veins that form areolae with included veinlets, is a genus of Asian ferns comprising approximately 40 species. *Cyrtomium* contains many apogamous species, such as *C. macrophyllum* Tagawa (Kurita 1967, Hirabayashi 1970), *C. caryotideum* C. Presl (Kurita 1966, Mitsuta 1986, Matsumoto 1976), and *C. fortunei* J. Sm. (Kurita 1960, Mitui 1968, Mitui 1980, Hirabayashi 1970, Matsumoto & Shimura 1985). In terms of reproductive modes and cytotypes of *C. fortunei*, Nakato *et al.* (1995) reported a diploid sexual type from China that has 82 somatic chromosomes and 64 spores in each sporangium, in addition to a triploid apogamous type, which has 123 somatic chromosomes and 32 spores in each sporangium. Until now, however, only apogamous triploids have been recorded in *C. fortunei* in Japan (Takamiya 1996).

In the most recent *Flora of Japan* by Iwatsuki (1992), *Cyrtomium fortunei s.lat.* was characterized by thin chartaceous to papyraceous pinnae with dentate distal margins, the number of pairs of pinnae usually more than 10, and pinnae less than 4 cm wide. Four varieties of *C. fortunei*, including var. *fortunei*, var. *clivicola*, var. *intermedium*, and var. *atropunctatum* have been recognized based on differences in (1) the number of pairs of pinnae, (2) the color of the indusium, and (3) the presence or absence of an auricle on the anterior base of the pinnae (Table 1). Among the four varieties of *C. fortunei*, var. *fortunei* has the highest number of pairs (15–30) of pinnae. Varieties *clivicola*, *intermedium* and *atropunctatum* have 5–20, 10–15, and 10–20 pairs, respectively. The color of the indusia of vars. *intermedium* and *atropunctatum* is grayish white with a dark brown center. The indusia in the other two varieties are completely grayish white. Var. *intermedium* has an auricle at the anterior base of each pinna, which is absent in var. *atropunctatum*. Vars. *fortunei* and *clivicola* sometimes have auricles, but not always.

The numbers of pairs of pinnae overlap among the varieties. Because morphological variation is continuous and individuals with intermediate morphology or various combinations of the above-mentioned morphological characteristics exist, it is very difficult to distinguish among the four varieties. Furthermore, even within local populations of *Cyrtomium fortunei* in Japan, large and continuous morphological variation is observed. Since *C. fortunei* is apogamous, it is expected to have low or discontinuous intraspecific variation, but the actual situation is very different from expectations.

Because it is difficult to identify the varieties of *Cyrtomium fortunei* on morphological characteristics, we sought to determine the genetic basis of their differences. Furthermore, it was important to clarify the relationship between morphological variation and genetic clones.

In the present study, fresh leaf samples were collected from mature individuals of *Cyrtomium fortunei* in four distantly located populations in Japan. In all populations, individuals that could be identified to the different varieties of *C. fortu-*

TABLE 1. Distinctive morphological characteristics of four varieties of *Cyrtomium fortunei sensu lato* in Japan.

	Number of pairs of pinnae	Color of center of indusium	Presence or absence of auricle at anterior base of pinnae
Cyrtomium fortunei			
var. *fortunei*	15–30	grayish white	present/absent
var. *clivicola*	5–20	grayish white	present/absent
var. *intermedium*	10–15	dark brown	present
var. *atropunctatum*	10–20	dark brown	present

nei grew together. We first determined the genotypes of all samples, then distinguished clones using the *rbcL* sequence and allozyme variation as genetic markers. In asexually reproducing organisms, the minimum biological unit is the genetic clone. We therefore concluded that the most efficient method of analyzing apogamous species such as *C. fortunei* was use genetic markers to identify clones growing within local populations. Such an intensive genetic analysis of a population of apogamous ferns has never been performed.

Allozyme variation is a powerful codominant marker for population genetic analyses of wild plants. Nucleotide sequence variation in *rbcL*, which is encoded in chloroplast DNA and is maternally inherited in ferns, is a useful tool for determining maternal races in local fern populations (Yatabe *et al.* 1998, Murakami *et al.*1999). Lu *et al.* (2005) sequenced the chloroplast DNA *rbcL* and *trnL–F* regions of 19 species of the *Cyrtomium*, including *C. fortunei*, and eight species of related genera to establish the molecular phylogeny. They did not, however, analyze the varieties of *C. fortunei*. The *rbcLs* from the four varieties of *C. fortunei* have not been analyzed. After identifying the genetic clones, we compared morphological variation between the same and different genetic types within local populations and between different populations.

Each genetic type that could be distinguished by *rbcL* and/or allozyme markers was tentatively recognized as a separate clone. It should be noted that genetic types differing in any genetic marker belong to different clones, but types with identical genotypes do not necessarily belong to the same clone.

In this study, the following four questions were specifically considered: (1) Do several different genetic clones exist in each population where several morphological varieties grow together? (2) If several genetic clones do exist in each local population, how many clones are present in each population? (3) Are the same clones (individuals showing the same genetic type) shared, even among remote populations, or are different clones restricted to a local population? (4) Are genetic differences observed among the varieties correlated with morphological characteristics, or do the four varieties correspond to different genetic clones?

Materials and Methods

Study sites and sample collections

Samples of *Cyrtomium fortunei* used in this study were collected from four populations in Japan: Moroyama, Saitama Pref.; Kawazu, Shizuoka Pref.; Kobe, Hyogo Pref.; and Fuchu, Hiroshima Pref. (Fig. 1). In each population, many morphological variants, which could be assigned to several different varieties of *C. fortunei*, grew together.

In general, whole fresh leaf samples were collected from mature individuals in each population. In the Moroyama, Kobe, and Fuchu populations, quadrats were established and the location of each individual was recorded before the samples were collected. In the Moroyama population, 51 mature individuals of *Cyrtomium fortunei* were examined in an area of 60 × 20 m. In the Kobe population, mature leaves were collected from 83 individuals in an area of 100 × 20 m and from an additional 24 nearby individuals. In the Fuchu population, leaf samples from 55 individuals were collected in an area of 50 × 50 m. Quadrat size differed among populations because quadrats were set so that the total number of samples in each quadrat was approximately 50, despite differing densities of individuals. The density of plants of *C. fortunei* in the Kawazu population was too low to establish a quadrat. In all populations, the environment, such as the amount of direct sunlight (amount of shade), moisture in the soil, and topographical features were observed and recorded for each sample.

We identified the four varieties of *Cyrtomium fortunei* on the basis of the distinctive morphological characters shown in Table 1. We tentatively identified individuals with more than 15 pairs of pinnae as var. *fortunei* and those with 15 or fewer pairs of pinnae as var. *clivicola*. *Cyrtomium fortunei* var. *clivicola* and var. *intermedium* were found in the Moroyama and Kobe populations, although those varieties were not always distin-

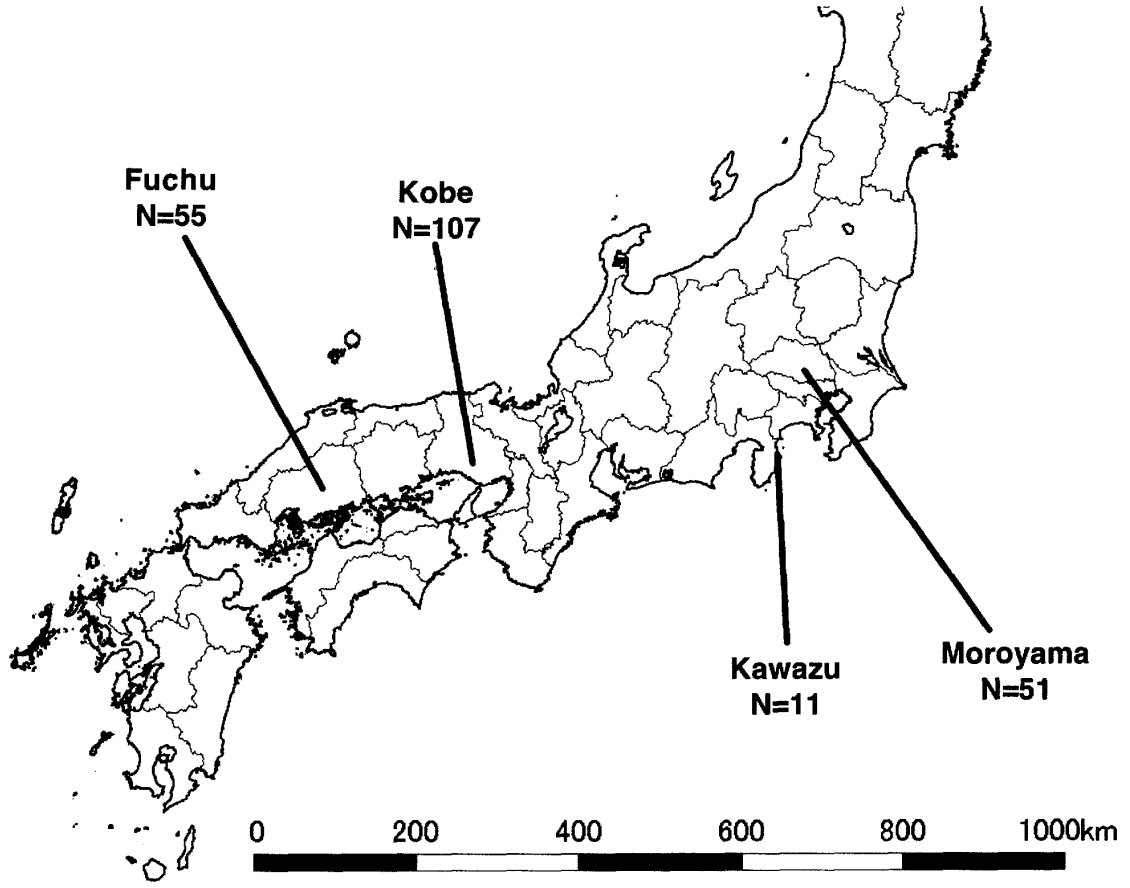

FIG. 1. Location of the four study sites and number of individuals examined in each population.

guishable due to continuity in their morphological variation. Only var. *fortunei* and var. *clivicolum* were in the Fuchu population; var. *intermedium* was not found. Var. *atropunctatum* was also in the Kawazu population, in addition to var. *fortunei* and var. *clivicola*.

Two pinnae were removed from each leaf sample. One pinna was maintained as a fresh leaf sample at 4°C in the refrigerator until it was used for allozyme and ploidy analyses. The other pinna was dried with silica gel and kept in a small plastic bag at room temperature until it was used for DNA extraction. Fresh root samples were collected from representative individuals in the Kobe population. The remaining portion of the leaves was dried in newspapers to serve as a voucher specimen. All voucher specimens have been deposited in the Makino Herbarium (MAK) of Tokyo Metropolitan University.

DNA extraction and sequencing of rbcL *gene*

Total DNA was extracted from silica gel dried leaf samples using 2× hexadecyltrimethylammonium bromide (CTAB) solution according to the method of Doyle and Doyle (1990) with some modifications. In brief, 100 mg of dried leaves were ground into a fine powder using a TissueLyser (Retsch QIAGEN, Germany). Next, 500 μL of 2× CTAB solution was added, mixed, and heated at 55°C for 20 min. After chloroform-isoamyl alcohol (24:1) extraction, isopropanol precipitation was performed. The DNA pellet obtained was washed with 70% ethanol, air dried, and redissolved in 50 μL TE buffer. PCR amplification of the *rbcL* gene was performed using Nova Taq Hot Start DNA Polymerase (Novagen, Madison, WI), 1× Ampdirect Plus Buffer (Shimazu, Kyoto, Japan), and the af and cr primers of Hasebe *et al.* (1994). In addition, four original internal primers dF (5′-GGTGTTGGATTCAAGCTGGT-3′), dR (5′-GAGCCTGTACGCAAGCTTCT-3′), e2F (5′-GCGGTGGACTTGATTTCACA-3′), and e2R (5′-GACAATTGGTGCACCCAACT-3′) were developed in this study and used for sequencing.

The conditions for PCR amplifications were as follows: initial denaturation at 95°C for 10 min, 45 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 90 s. The PCR products were incubated at 37°C for 30 min and at 80°C for 20 min with 5% ExoSAP-IT (USB, Cleveland, Ohio) to remove single-stranded DNA. For cycle sequencing reactions, a BigDye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA) was used. The reaction mixtures were analyzed on an automated DNA sequencer model 3100 (Applied Biosystems). The nucleotide sequences obtained were aligned using ClustalX2 (Larkin *et al.* 2007).

Molecular phylogenetic analysis

To compare the *rbcL* sequences obtained from Japanese *Cyrtomium fortunei* with those obtained by Lu *et al.* (2005) from Chinese plants, maximum parsimony (MP) analysis was performed using MEGA 5 software (http:// www.megasoftware.net/). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with random addition of sequences (10 replicates). Analysis involved the 2 nucleotide sequences of *rbcL* from Japanese plants as well as the 16 *rbcL* sequences reported by Lu *et al.* (2005) from *C. macrophyllum*, *C. omeiense*, *C. urophyllum*, *C. caryotideum*, *C. aequibasis*, *C. fortunei*, *C. devexiscapulae*, *C. falcatum*, *C. nephrolepioides*, *C. shingianum*, *C. hemionitis*, *C. grossum*, *C. chingianum*, *C. lonchitoides*, and *C. guizhouense* from China. A total of 1237 positions were in the final dataset of the *rbcL* sequences. *Polystichum lonchitis* was used as an outgroup. Bootstrap analysis with 1000 replicates was performed to evaluate internal support for the trees obtained.

Allozyme analysis

Fresh pinnae were ground in 1 ml of cold extraction buffer (pH = 7.5) containing 0.1 mM Tris-HCl, 1 mM EDTA-4Na, 10 mM KCl, 10 mM $MgCl_2$, 0.4% 2-mercaptoethanol, and 10% polyvinylpyrrolidone. Enzymes were resolved on 6% polyacrylamide gels following the procedures of Shiraishi (1988). We examined phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), hexokinase (HK), and leucine aminopeptidase (LAP), following the procedures of Shiraishi (1988). Loci were numbered, with the most anodal locus as *1* and progressively more cathodal loci with higher numbers. Alleles were similarly indicated at each locus, with the most anodal form designated "*a*" and progressively slower forms as "*b*," "*c*," and so on.

Estimation of reproductive mode

The reproductive mode (apogamous or sexual) in most homosporous ferns can be estimated simply by counting the number of spores in a sporangium. Sexual ferns usually have 64 spores per sporangium, whereas apogamous ferns have 32 (Manton 1950). We counted the number of spores per sporangium in the voucher specimens from the above four localities to determine the reproductive mode.

Cytological observation and ploidy analysis using flow cytometry

Mitotic chromosomes were observed in materials collected from six plants, which showed different genetic types, in the Kobe population. In this population, the largest number of different genetic types was identified by *rbcL* sequencing and allozyme analysis. Therefore, cytological observations and ploidy analyses of *Cyrtomium fortunei* were performed on this population. Root tips were pretreated with 0.002 M 8-hydroxyquinoline for 6 h at approximately 20°C. After fixation in 45% acetic acid for 15–30 min, the root tips were hydrolyzed in 1 N HCl at 60°C and then squashed in 2% aceto-orcein. Chromosomes were observed with an Olympus BX-41 microscope and photographed with an Olympus DP-50 digital camera.

Furthermore, to determine the ploidy level of individuals growing in the Kobe population in detail, the DNA content of each nucleus extracted from fresh pinnae samples was measured by flow cytometry using a CyFlow Ploidy Analyzer (Partec, Munster, Germany) as well as a Cystain UV Precise P kit (Partec, Munster, Germany). A

sample leaf segment approximately 5 × 5 mm was finely chopped with a razor blade in 0.5 ml of nucleus extraction buffer from the kit, then filtered through a 50 μm mesh and stained with 1.5 ml of staining buffer containing 4′, 6′-diamidino-2-phenylindole hydrochloride from the kit. Fresh leaf tissues of a diploid individual of *C. fortunei* collected in China (Nakato *et al.* 1995) and another triploid individual from the Kobe population, whose ploidy level was determined from chromosome counts during this study, were used as controls. First, the DNA content per nucleus was measured for the diploid and triploid controls. We then analyzed 80 samples collected from the Kobe population under the same conditions.

Morphological observation

After identifying the allozyme types, morphological characters among the different genetic types were analyzed and compared. The characters used were (1) number of pairs of pinnae, (2) color of indusium, and (3) presence or absence of an auricle on the anterior base of the pinna. Differences in the characters (1) among the allozyme types were statistically tested using the Steel-Dwass' test.

Quantification of genetic variation

To quantify the clonal diversity detected in each population as well as within *Cyrtomium fortunei*, Simpson's diversity index (*D*) was calculated for every population as well as for the entire species:

$D = 1-\Sigma\{[n_i(n_i-1)]/[N(N-1)]\}$,

where n_i is the number of individuals of variant i and N is the number of individuals collected.

Results

Nucleotide sequence variation of rbcL *gene*

We determined the 1317-bp nucleotide sequence of the *rbcL* gene for 224 individuals of *Cyrtomium fortunei* from the four populations. Two types of *rbcL* sequences were obtained, hereafter referred to as *rbcL* sequences α and β. The sequences have been deposited in the DNA Database of Japan under accession numbers AB598689 and AB598690, respectively. Seven base pair substitutions were observed between the two sequences. Leaf samples collected from the Moroyama and Kobe populations showed both α and β *rbcL* sequences (Table 2). In contrast, samples collected from the Kawazu and Fuchu populations showed only sequence α.

Molecular phylogenetic analysis

Molecular phylogenetic analysis of *rbcL* sequences obtained from *Cyrtomium* was performed by the MP method to infer the phylogenetic position of the sequences α and β. One of the 30 most parsimonious trees (length = 78) obtained in this study is shown in Fig. 2 with bootstrap probabilities. Its consistency index was 0.72 and retention index was 0.80. The *rbcL* sequence β from Japanese *C. fortunei* was the same as in *C. aequibasis*. *Cyrtomium aequibasis* is more closely related to *C. macrophyllum*. *rbcL* sequence α differed in two base pairs from the Chinese *C. fortunei* reported by Lu *et al.* (2005), but still formed a clade with it (Fig.2).

Allozyme variation

We performed electrophoretic analysis of four enzyme systems and determined the genotype of 224 individuals of *Cyrtomium fortunei*. We were able to resolve six loci in total. For PGM and 6PG enzyme systems, two loci (*Pgm-1* and *Pgm-2*, and *6pg-1* and *6pg-2*) were detected, although *6pg-1* showed no allelic variation and was excluded from further analysis. For HK and LAP enzyme systems, one locus each was detected.

In total, eight electrophoretically distinguishable types were recognized (Fig. 3 and Table 2) and named allozyme types A through H. Genotypes and the number of individuals of each allozyme type observed in the four populations examined are shown in Table 2. In all four populations examined in this study, individuals with several allozyme types were observed, indicating that several different clones were growing together in each population (Fig. 4). In the Moroyama and Kobe populations, three and five types were identified, respectively. Three allozyme types (A,

TABLE 2. Distribution of the two *rbcL* types (a and b) in four populations of *Cyrtomium fortunei s.lat.*

Population	*rbcL*-type		Total number of individuals examined
	α	β	
Moroyama	28	23	51
Kawazu	11	0	11
Kobe	85	22	107
Fuchu	55	0	55

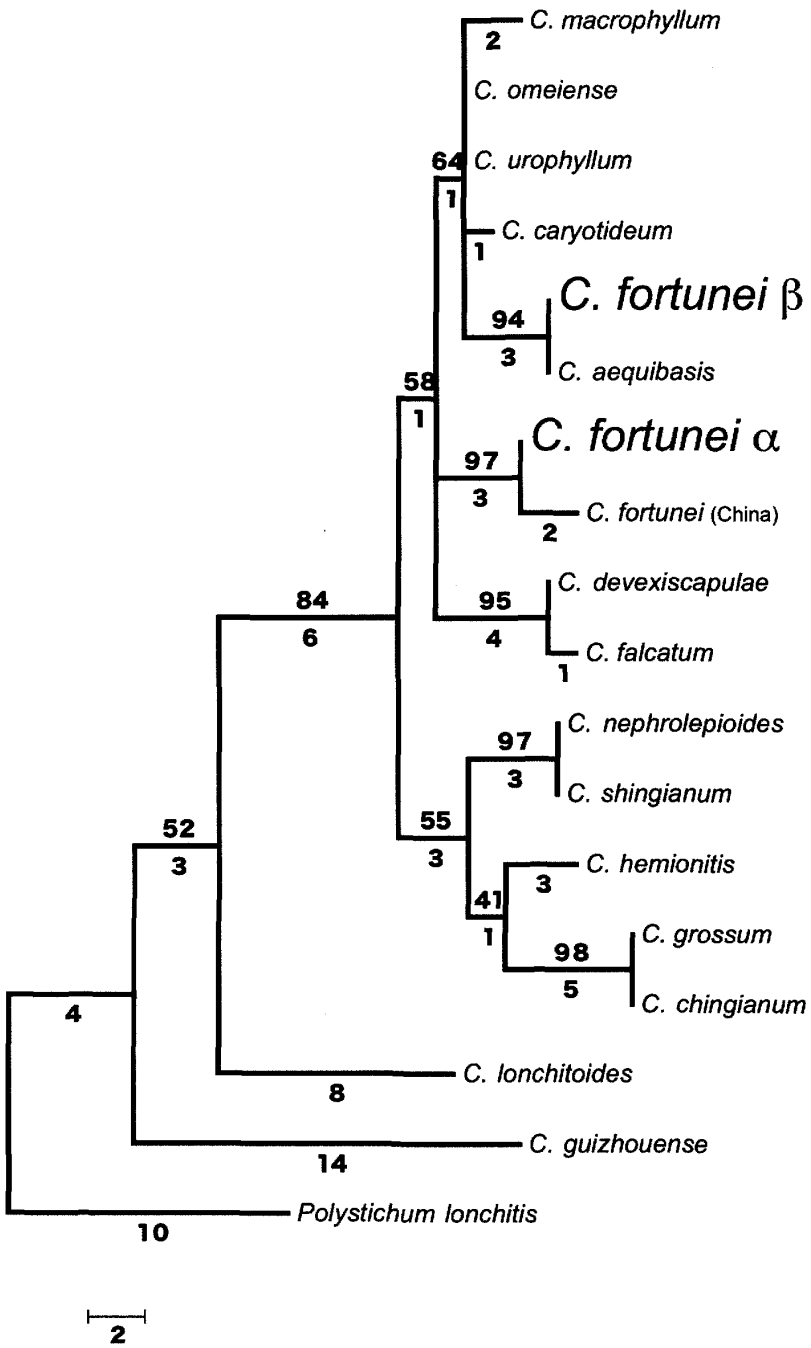

FIG. 2. One of the most parsimonious trees based on *rbcL* sequence of Japanese *Cyrtomium fortunei* and Chinese species of *Cyrtomium* reported by Lu *et al.* (2005) (length = 78 steps; consistency index = 0.72; retention index = 0.80). Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) and number of base pair substitution are shown above and below each branch, respectively.

E, and H) were in common between these two populations, although the populations are remote, separated by approximately 500 km (Table 3). Only individuals of allozyme type H had *rbcL* sequence β, whereas those of all other allozyme types had *rbcL* sequence α. Table 3 summarizes the results of Simpson's diversity index *(D)*.

Reproductive mode

Reproductive mode was estimated by counting the number of spores per sporangium for 140 individuals (46 from the Moroyama population; 11 from Kawazu; 46 from Kobe; and 43 from Fuchu). At least two individuals in each of the eight clones recognized in this study were included in the analyses. All individuals examined had 32 spores per sporangium, suggesting that plants of all eight allozyme types were apogamous.

Morphology

From our genetic analyses and morphological observations we found that all individuals of allozyme type H with *rbcL* sequence β had bicolored indusia (Fig. 5). Plants with allozyme type B in the Kawazu population had bicolored indusia, but their *rbcL* sequence was α. Individuals of all other allozyme types (A, C–G) had uniformly light gray indusia, although they varied in leaf morphology.

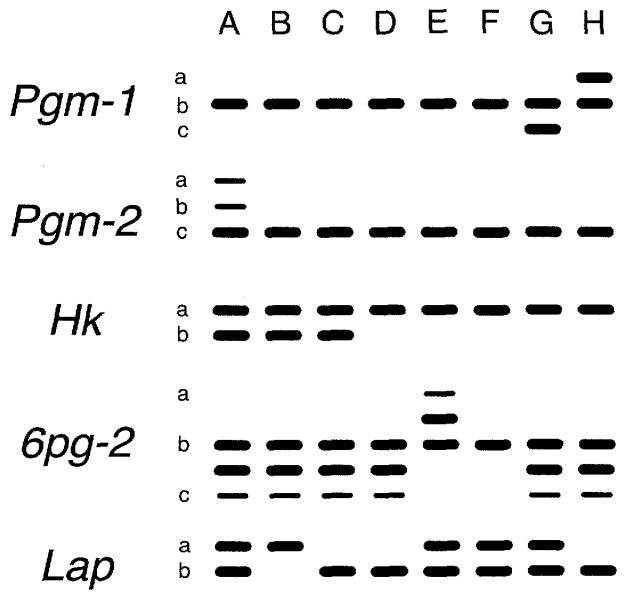

FIG. 3. Zymograms of Japanese *Cyrtomium fortunei s.lat.* Band patterns of four enzyme systems (PGM, HK, 6PG, and LAP) are shown for each of the eight allozyme types (A–H).

The relationship between allozyme types and number of pairs of pinnae is shown in Table 4. In addition, variation in number of pairs of pinnae in each allozyme type is shown in Fig. 6. In Fig. 6 it can be seen that allozyme type D has a significantly greater number of pinnae than other allozyme types (A, B, C, E, F and H). Type G, however, was not significantly different from types A to F, including type D. Type H tended to have a lower number of pinnae, but not significantly less than in other types (A, B, C, E and F). Large variation in the number of pairs of pinnae was observed in all allozyme types, although it is still possible that several different clones were contained in one allozyme type. The relationship between the varieties of *Cyrtomium fortunei* and allozyme types is shown in Table 5.

Cytological analysis

The chromosomes of a few individuals of each of the five allozyme types from the Kobe population were counted (Fig. 7). All allozyme types showed $2n$ = ca. 123. All genetic types detected in the population were therefore determined to be triploid.

Flow cytometry showed that the triploid control had 1.5 times more DNA per nucleus than the diploid control. This diploid individual of *Cyrtomium fortunei* from China had *rbcL* α. Thus, its close relationship to the Japanese triploid *C. fortunei* could be supposed. The DNA content per nucleus in the 80 individuals from the Kobe population and in the triploid control was identical, and 1.5 times the nuclear content of the diploid control. All 80 individuals were therefore determined to be triploid. We concluded that all individuals of *C. fortunei* in the Kobe population are apogamous triploids.

Discussion

In this study, two *rbcL* types (α and β) and eight allozyme types (A–H) were detected in *Cyrtomium fortunei*. On the basis of morphology, four varieties were identified, however, a greater

TABLE 3. Distribution of eight allozyme types in four populations of *Cyrtomium fortunei s.lat.* and Simpson's diversity index (*D*) calculated on the basis of allozyme variation in each population. Individual numbers of each allozyme type found in the four local populations as well as values of *D* are shown.

Population	Allozyme type								Simpson's Index
	A	B	C	D	E	F	G	H	
Moroyama	1				27			23	0.53
Kawazu		6	5						0.55
Kobe	37				26	11	11	22	0.77
Fuchu				51	2		2		0.14
Total	38	6	5	51	55	11	13	45	0.82

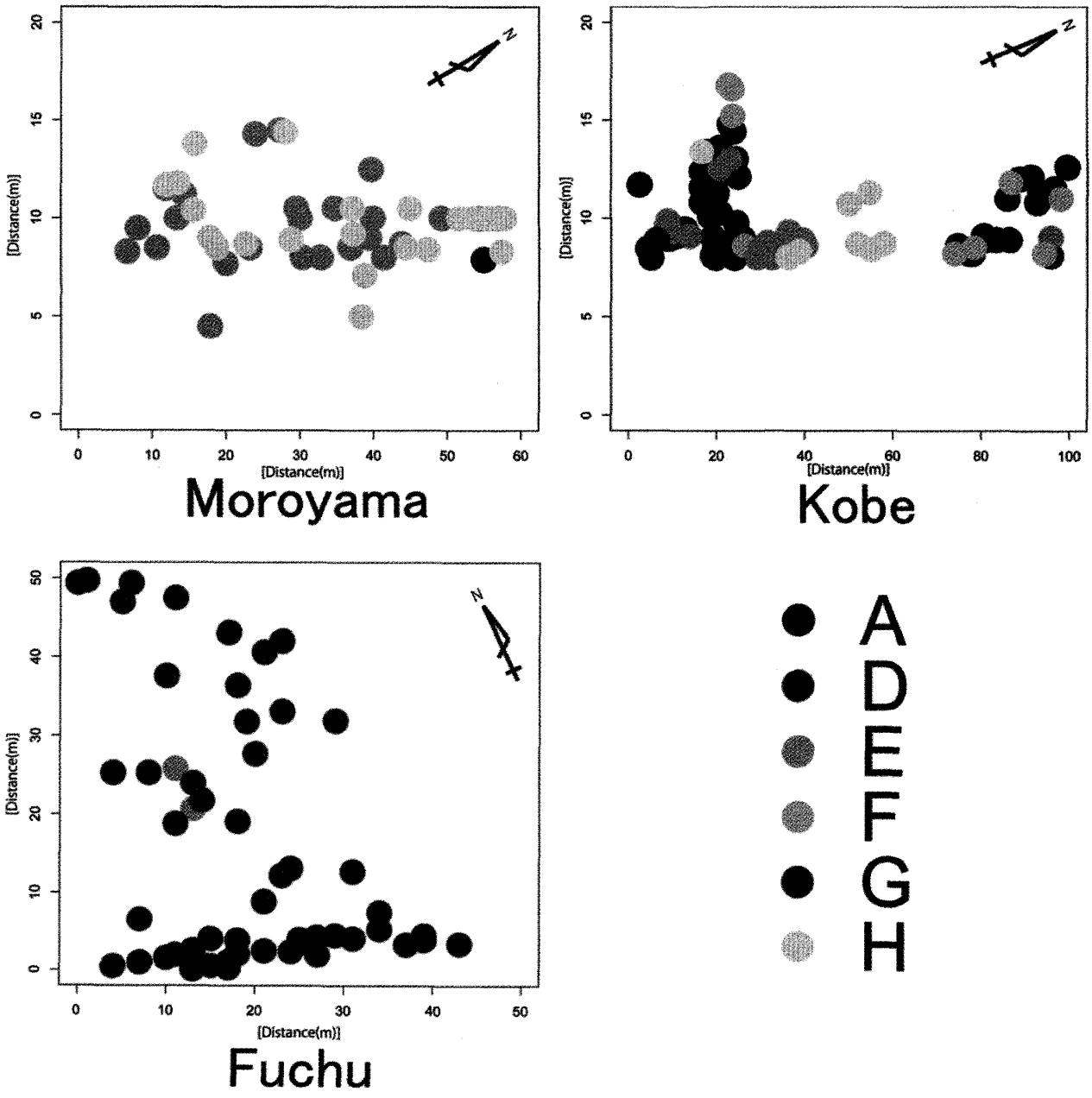

FIG. 4. Maps showing distribution of the eight allozyme types in the Moroyama (Saitama Pref.), Kobe (Hyogo Pref.) and Fuchu (Hiroshima Pref.) populations.

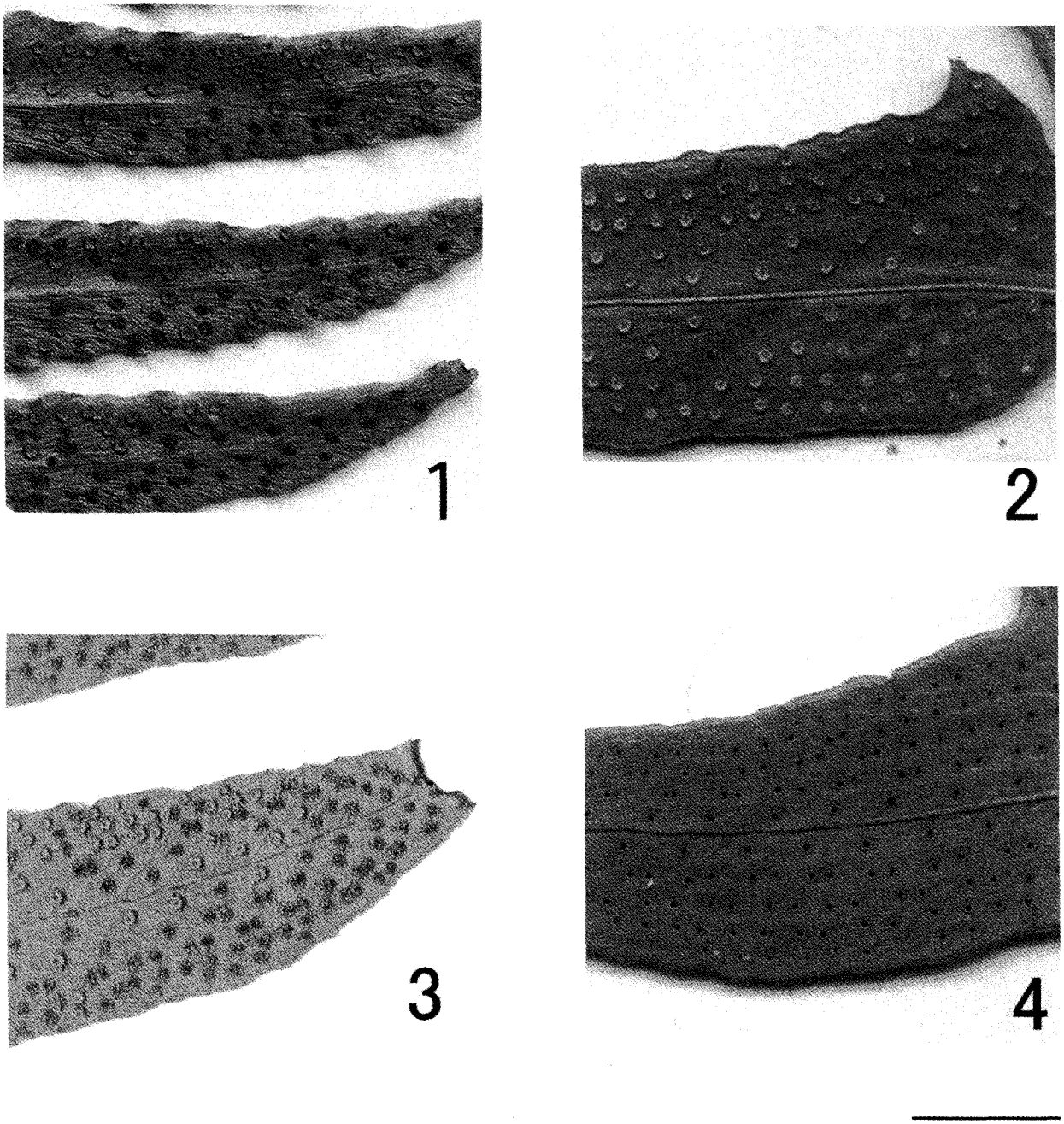

FIG. 5. Indusia of the four varieties of *Cyrtomium fortunei*. 1: var. *fortunei*. 2: var. *clivicolum*. 3: var. *atropunctatum*. 4: var. *intermedium* (bar = 1 cm).

number of genetic clones were observed in *C. fortunei*. In the Kobe and Moroyama populations, individuals of allozyme type H had *rbcL* sequence β, whereas those of all the other types had *rbcL* sequence α, thereby showing that *rbcL* β type was genetically different from the *rbcL* α type even in the nuclear genome (allozyme loci). Furthermore, only individuals with *rbcL* β and the H allozyme pattern had bicolored indusia, which is one of the morphological characteristics peculiar to *C. fortunei* var. *intermedium*. It was therefore concluded that *C. fortunei* var. *intermedium* is genetically different in its nuclear and plastid genomes from other varieties of *C. fortunei*. Seven base pairs were different between *rbcL* α and β. The differences were as large as those between distinct species (Yatabe *et al.* 2009). Zymograms of the H type are similar to zymograms of the other types (Fig. 3). To clarify the origin of var. *intermedium*, careful comparison with mem-

bers of the *Cyrtomium macrophyllum* group with *rbcL* β using several molecular markers is necessary.

As for other allozyme types, type B corresponded to *Cyrtomium fortunei* var. *atropunctatum*, which is also characterized by having bicolored indusia, although the color of their central portion is not as dark as in allozyme type H or var. *intermedium* (Fig. 5). Vars. *fortunei* and *clivicola* both have grayish white indusia, but we could not separate the allozyme types A, C, D, E, F and G into two groups so as to correspond to those two varieties.

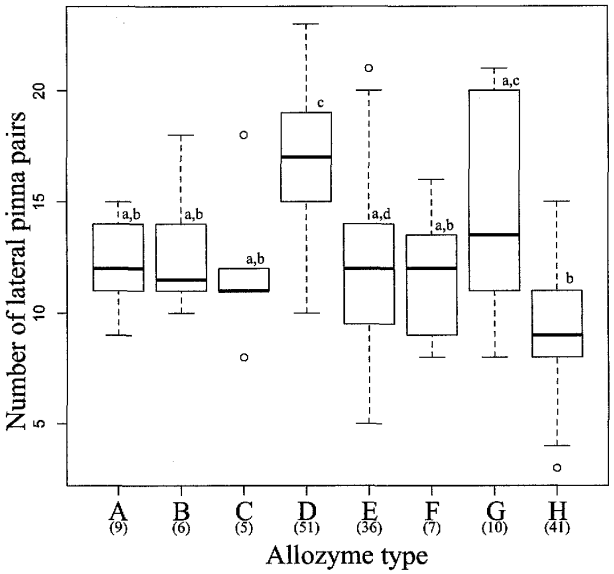

FIG. 6. Variation in number of pairs of pinnae in each allozyme type. Horizontal lines indicate minimum, first quartile, median (thick line), third quartile and maximum, respectively from bottom. Circles (○) indicate outlier points. Sample size is in parentheses. Letters (a, b, c and d) indicate significant difference at $P < 0.05$ by Steel-Dwass' multiple range test.

On the basis of our *rbcL* and allozyme data, *Cyrtomium fortunei* vars. *fortunei* and *clivicola* were found to be morphologically and genetically indistinguishable. Using genetic markers, we determined that at least seven genetic clones were present in the apogamous *Cyrtomium fortunei*, even after var. *intermedium*, which is genetically and morphologically distinct, was excluded from it. How did an apogamous species acquire such considerable genetic variation? Several hypotheses have been proposed to explain increased genetic variation within an apogamous fern: (1) hybridization with closely related sexual species (Suzuki & Iwatsuki 1992), (2) unequal meiosis (Lin *et al.* 1992), (3) recurring origin (Watano & Iwatsuki 1988), and (4) genetic segregation by homoeologous chromosome pairing (Klekowski 1973, Ishikawa *et al.* 2003a, b). Hypotheses (1) and (2) cannot explain the genetic variation observed in *C. fortunei* because only apogamous triploids have been recorded in *C. fortunei* in Japan. In *C. fortunei*, hypothesis 3, the recurring origin of triploid apogamous types from related sexual types in the past, remains. It seems, however, that genetic variation would not be main-

TABLE 4. Relationship between number of pairs of pinnae and allozyme types.

Number of pairs of pinnae	Allozyme type							
	A	B	C	D	E	F	G	H
1– 5	0	0	0	0	2	0	0	2
6–10	2	1	1	1	8	3	2	28
11–15	7	4	3	17	21	3	4	11
16–20	0	1	1	22	4	1	2	0
20–25	0	0	0	11	1	0	2	0

TABLE 5. Relationship between morphological variety and allozyme type.

Varieties	Allozyme type							
	A	B	C	D	E	F	G	H
var. *fortunei*	○		○	○	○	○	○	
var. *clivicola*	○		○	○	○	○	○	
var. *intermedium*								○
var. *atropunctatum*		○						

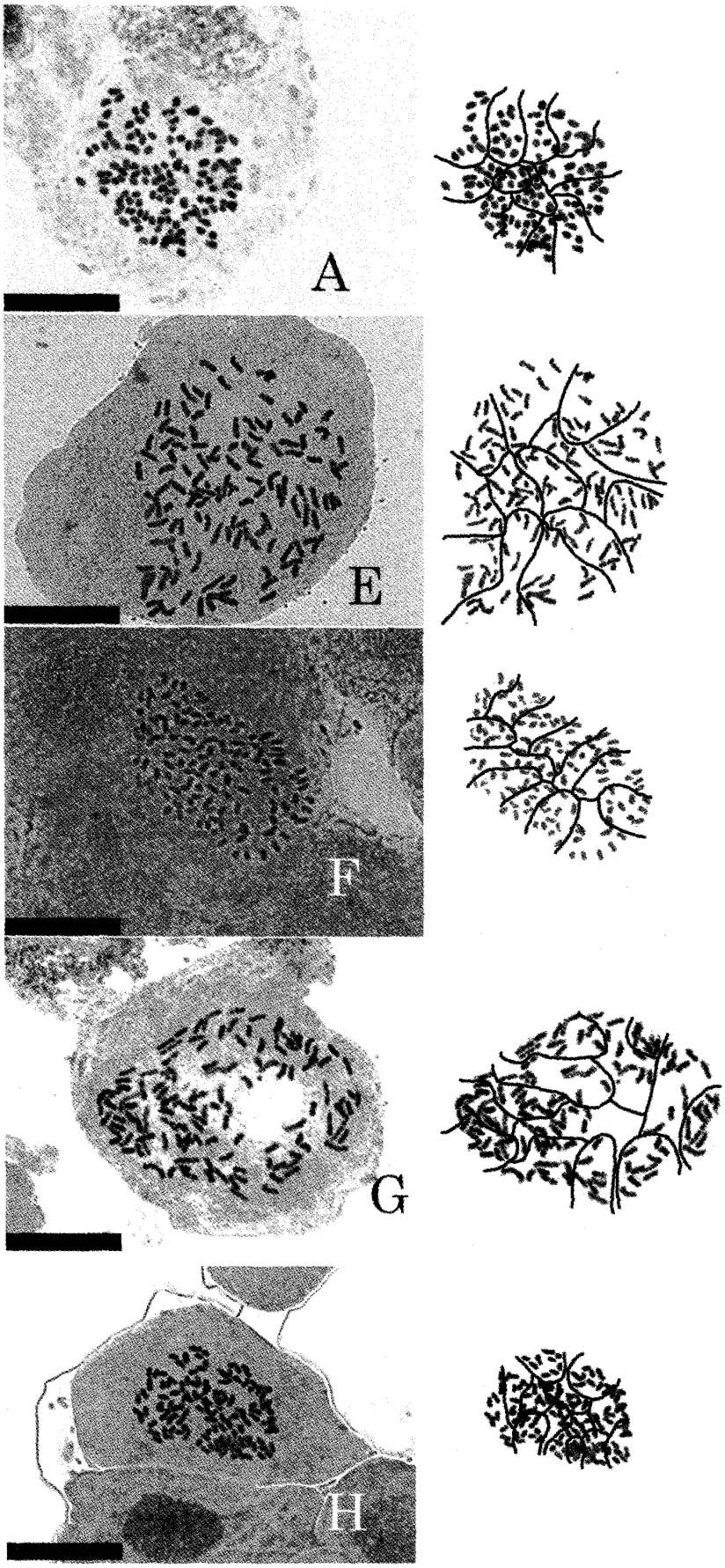

FIG. 7. Somatic chromosomes of individuals with allozyme types A, E, F, G, and H in the Kobe population (bar = 50 μm), and their explanatory diagrams (right). All clones had $2n$ = ca. 123.

TABLE 6. Comparison of Simpson's diversity index (*D*) calculated on the basis of allozyme variation in six apogamous species *Cyrtomium fortunei*, *Dryopteris bissetiana*, *D. nipponensis*, *D. pacifica*, *D. sacrosancta* and *D. varia*.

Species	Simpson's Index D	Reference
C. fortunei	0.82	This study
D. bissetiana	0.81	Lin *et al.* (1995)
D. nipponensis	0.81	Ishikawa *et al.* (2003a)
D. pacifica	0.91	Lin *et al.* (1995)
D. sacrosancta	0.33	Lin *et al.* (1995)
D. varia	0.90	Lin *et al.* (1995)

tained for long periods (thousands of generations) in local populations, usually consisting of at most several hundreds individuals. Natural selection and genetic drift inevitably operate, even in populations of apogamous species. Moreover, gene flow among populations does not appear high, as shown by the considerable differences in constitution and frequencies in allozyme types among the populations. As a result, several different clones do not appear to be maintained in apogamous species unless genetic variation is constantly generated. We therefore consider novel genetic clones to be produced from an apogamous lineage of *C. fortunei*.

In the Kobe population, a series of band patterns were observed on zymograms obtained from the samples that could have been easily generated through segregation during homoeologous chromosome pairing. For example, band patterns of clone F can be explained by segregation from those of clone E, especially at the *6pg-2* loci. The frequency of segregation by homoeologous paring in *C. fortunei* should be examined in future studies. For this purpose, we need to analyze and compare the genetic composition of offspring derived from an individual parent in an apogamous species.

If genetic segregation by homoeologous chromosome pairing occurs in an apogamous fern, it is expected that homozygosity will increase and heterozygosity will disappear. Heterozygous allozyme patterns, however, were observed in *Cyrtomium fortunei*. We considered that deleterious genes were expressed in some of the segregated individuals and were removed from the populations.

Another interesting question related to the relatively high genetic variation in *Cyrtomium fortunei* is how genetic diversity is maintained in many local populations of apogamous fern species. The value of Simpson's diversity index *(D)* calculated for *C. fortunei* was high enough, even compared with *Dryopteris nipponensis* and *D. pacifica*, in which genetic segregation and unequal meiosis were reported, respectively (Table 6). This suggests that several different clones grow together in each local population of *C. fortunei*. Two hypotheses can explain this situation: (1) niche differentiation occurs among different clones. Several clones can therefore coexist for long periods of time within each population, and (2) new clones are always generated by mutation and/or genetic recombination faster than they are removed from the population by selection and/or random genetic drift. The first hypothesis predicts that each clone grows in different environments. In fact, niche differentiation, or at least diversity related to the amount of shade and soil moisture among allozyme types, was not observed in this study, although we located each genetic type in the three different local populations. The second hypothesis predicts that new clones with different genotypes will be generated at a relatively high rate. These two hypotheses are not mutually exclusive, but more detailed ecological comparison among the allozyme types in each population and examination of the frequency of genetic segregation is necessary to understand the high genetic variation observed in populations of the apogamous *C. fortunei*.

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